UNIVERSIDAD AUTÓNOMA DE GUERRERO
UNIDAD ACADÉMICA CIENCIAS QUÍMICO BIOLÓGICAS
UNIDAD ACADÉMICA DE MEDICINA
MAESTRÍA EN CIENCIAS BIOMÉDICAS

"POLIMORFISMOS EN EL GEN DE LA γ-GLUTAMIL HIDROLASA Y RESPUESTA AL METOTREXATO EN LEUCEMIA LINFOBLÁSTICA AGUDA EN MÉXICO"

T E S I S

QUE PARA OBTENER EL GRADO DE

MAESTRÍA EN CIENCIAS BIOMÉDICAS

P R E S E N T A :

JORGE ORGANISTA NAVA

DIRECTOR DE TESIS: **DR. MARCO ANTONIO LEYVA VÁZQUEZ**

CHILPANCINGO GRO., AGOSTO DE 2009.

UNIVERSIDAD AUTÓNOMA DE GUERRERO
UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS
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APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 10 días del mes de julio de dos mil nueve, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Ciencias Biomédicas, para examinar la tesis titulada **"Polimorfismos en el gen de la γ-glutamil hidrolasa y respuesta al metotrexato en leucemia linfoblástica aguda en México"**, presentada por el alumno Jorge Organista Nava, para obtener el Grado de **Maestría en Ciencias Biomédicas**. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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Polymorphisms in the γ-glutamyl hydrolase gene and response to methotrexate in acute lymphoblastic leukemia in Mexico

Este trabajo se realizó en el Laboratorio de Biomedicina Molecular de la Unidad Académica de Ciencias Químico-Biológicas de la Universidad Autónoma de Guerrero, en la Ciudad de Chilpancingo Gro, México.

Bajo la dirección de

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Instituto Estatal de Cancerología "Arturo Beltrán Ortega"
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Dra. Ana Bertha Rivera Ramírez

Durante el período en que cursó la Maestría en Ciencias Biomédicas, el C. Jorge Organista Nava, recibió beca del CONACYT.

El presente trabajo fue enviado para su publicación a la revista **Leukemia Research,** por lo que el contenido de la tesis corresponde al formato de la editorial correspondiente.

AGRADECIMIENTOS

A DIOS:

Por permitirme vivir este día y estar con mis seres amados. También por la fuerza que me has dado.

A MIS PADRES Y HERMANAS

Por su cariño, su forma de educarme, sin imponerme nada, sin exigirme nada, dándome siempre libertad para que hiciera lo que consideraba oportuno, pero a la vez dándome unos valores que son el mayor tesoro que poseo. También de mis hermanas, que siempre han estado apoyándome. Gracias!!

A YAZMÍN

Por todo el apoyo, la confianza y la moral que necesitaba y por darme valor para enfrentar los obstáculos que se me presentaron y por apoyarme en todas las decisiones que tome y las que seguiré tomando.

A MIS AMIGOS

Que siempre han estado allí, por los momentos maravillosos que he pasado y espero pasar en su compañía. Los que tengo desde la infancia, los que conocí durante la carrera y aquellos más recientes que he conocido en la maestría. Tengo la suerte de que son muchos, así que no voy a dar sus nombres, pero ellos saben quiénes son.

A MIS COMPAÑEROS Y AMIGOS DEL LABORATORIO

Q.B. P. Natividad Sales Linares, M.C. Verónica Antonio Vejar, M.C. Noelio Zamudio López y M.C. Miguel Ángel Rodríguez Barrera. Por todas sus aportaciones, observaciones y sobre todo por ayudarme a salir adelante con mi tesis.

A MIS MAESTROS

Con profundo agradecimiento a mis maestros, por compartir conmigo sus conocimientos y experiencias durante el viaje que emprendimos juntos. A la Dra. Elba Reyes Maldonado, Dra. Luz del Carmen Alarcón Romero, MC. Ma. Elena Moreno Godínez, MC. Adolfo Román Román, Dra. Gloria Fernández Tilapa por su profesionalismo y por sus valiosos comentarios y sugerencias durante el desarrollo del trabajo de tesis.

A la Dra. Berenice Illades Aguiar y al Dr. Marco Antonio Leyva Vázquez porque me permitieron estar en este proyecto de tesis, a si como todo su apoyo incondicional y depositar su confianza en mí. Espero que todo el tiempo que les he robado haya merecido la pena.

Abusando aún más de gente tan maravillosa, les pido que en esta nueva etapa de mi vida que ahora comienza, sigan estando conmigo, apoyándome y ayudándome como lo han hecho hasta ahora.

JORGE ORGANISTA NAVA

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Manuscript Received

De: "Leukemia Research" leukemiaresearch@urmc.rochester.edu

Para: leyvamarco@yahoo.com.mx

Enviados: Sábado, 27 de Junio 2009 12:15:02 GMT -08:00

Asunto: Leukemia Research Manuscript Received

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Article Title: Polymorphisms in the □-glutamyl hydrolase gene and response to methotrexate in acute lymphoblastic leukemia in Mexico.

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Polymorphisms in the γ-glutamyl hydrolase gene and response to methotrexate in acute lymphoblastic leukemia in Mexico

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ABSTRACT

This study evaluated the association of -401C/T and +452C/T polymorphisms of γ-glutamyl hydrolase and the lack of response to methotrexate in acute lymphoblastic leukemia. Genotyping was performed 70 children with acute lymphoblastic leukemia and 140 without. Association between -401C/T polymorphism and lack of response was found (p=0.028), patients with -401T/T genotype have 10.83 (CI95% 1.30-90.14) more chance for a relapse of leukemia. No association was found between the +452C/T polymorphism and the response. Therefore, our investigation suggests that the -401C/T polymorphism in the γ-glutamyl hydrolase may be a factor that would generate this lack of response to methotrexate in patients with ALL while the +452C/T cannot.

Keywords: acute lymphoblastic leukemia, γ-glutamyl hydrolase, lack of response to methotrexate, single nucleotide polymorphism, -401C/T polymorphism, +452C/T polymorphism.

1. INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most frequent type of cancer in children worldwide [1-3]. Mexico City has seen a significant increase in the incidence of ALL. Data from 1996 to 2000 show a rate of 63.7 per million children, one of the highest rates reported in the world. The National Institute of Statistics, Geography and Computing (INEGI) and the General Direction of Information in Health of the Secretary of Health, published that in the State of Guerrero, in the year 2005, leukemia occupied the second cause of death in children less than 15 years of age [4].

For five decades, methotrexate (MTX) has been an essential drug in treatment regimens for patients with ALL and is presently used in the treatment of a number of other neoplastic diseases, including osteogenic sarcoma, breast cancer, head and neck cancers, and non-Hodgkin's lymphoma [5-8]. MTX efficacy is limited due to inherent and acquired resistance during treatment. However, approximately 80% of ALL children experience good clinical response [9-11]. After its entry into cells, MTX is rapidly converted to γ-glutamyl polyglutamates (MTXPG) through the action of folylpolyglutamate synthetase, which sequentially adds up to six glutamyl residues to MTX. MTX-PG directly blocks folate-dependent enzymes that lead to inhibition of *de novo* thymidine and purine synthesis, arresting DNA replication and causing cell death [10, 12].

γ–glutamyl hydrolase (GGH, also known as folypolyglutamate hydrolase, FPGH, EC 3.4.19.9) is a peptidase that catalyses the removal of γ-linked polyglutamates, converting long-chain MTX polyglutamate (MTXPG) into short-chain MTXPG and ultimately to MTX, causing the dimished therapeutic activity of MTX [13-15]. Several reports have shown that single nucleotide polymorphisms (SNPs) play an important role in the response to MTX treatment in patients with ALL [16-18]. GGH is altered by its polymorphisms, while the +452C/T polymorphism in codon 127 modifies the catalytic and hydrolytic activities of the enzyme on MTXPG in cells from B or T lineage, the -401C/T polymorphism is associated with resistance to MTX in patients with ALL [19-23].

Frequencies of the -401 C/T and +452C/T polymorphisms in the γ-glutamyl hydrolase gene in American, Korean, European, Caucasian and African-American populations have reported, however, in Mexico there are no reports of -401C/T and +452C/T polymorphisms in the γ-glutamyl hydrolase gene in children with ALL so a possible association with lack of

response to methotrexate cannot be made. The objective of this study was to assess the association of -401C/T and +452C/T polymorphisms in the γ-glutamyl hydrolase gene with lack of response to MTX treatment in ALL children. Our results suggest that the -401C/T polymorphism of GGH has to be determined to monitor efficacy and side effects of MTX in ALL patients.

2. MATERIAL AND METHODS

The study was carried out in the pediatric oncology service of the Cancer Institute of the State of Guerrero, Mexico, in the period from August 2005 to December 2008. We studied 70 children with a diagnosis of ALL by bone marrow aspirate based on French-American-British morphological criteria and cytochemical assays, treated with MTX according to the protocols 96091, 96092 or CIE-10: C9.1.0 of the Cancer Institute of the State of Guerrero [24] and those whose treatment changed for having had a relapse of ALL (> 25% of blasts in bone marrow after administration of MTX). We also included 140 children without ALL, with normal numbers of leukocytes (4.0-10 X 10^3 leucocytes/mm^3) and without a family history of leukemia. The age range of the participants was from 1-18 years and all were residents of the State of Guerrero. Informed consent was obtained from all the individuals or their guardians, after a detailed briefing of the study purposes. This study was approved by the ethics committee of the Institute of Cancer of the State of Guerrero.

2.1 Sample collection and DNA extraction

Peripheral blood samples were taken from all participants and collected in tubes with anticoagulant (EDTA), the extraction of genomic DNA from leukocytes was made by the phenol-chloroform technique [25, 26].

2.2 Genotyping of genetic polymorphisms

The -401C/T (ddsSNP; rs3758149) polymorphism were detected using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method reported by Dervieux *et al*. with some modifications [27]. Genomic DNA (100 ng) was amplified in 1X PCR buffer containing 200 μM of each dNTP (Applied Biosystems, Foster City, CA), 0.4 μMol each of forward primer (5'-CGCTGCCTGGTTACCAAACT-3') and reverse primer (TGTTTACGTCGATGTG GACTTCAG-3'), 0.5 units Taq DNA polymerase, Recombinant (Invitrogen™ life technologies, USA), and 1.5 mM of $MgCl_2$, in a final volume of 25 μl. PCR conditions were an initial denaturation step of 5 min at 95° C, 40 cycles of 15 s at 94° C, 45 s at 60° C, 45 s at 72° C, and a final extension step of 10 min at 72° C. The amplified PCR products (109 pb) were subjected to enzymatic digestion at 55° C using 2.5 U *Bsl* I (New England Biolabs, Berverly, MA, U.S.A.). Digested products were analyzed on 10% acrylamide gel [28, 29]. Individuals with the -401CC genotype presented two fragments (61

and 48 pb), individuals with the -401CT genotype presented three fragments (109, 61 and 48 pb) and those with the -401TT presented one fragment (109 pb).

The +452C/T polymorphism (ddsSNP; rs11545078) was detected using a PCR-RFLP method reported by Chave *et al.* with some modifications [19]. Genomic DNA (100 ng) was amplified in 1X PCR buffer containing 200 μM of each dNTP (Applied Biosystems, Foster City, CA), 0.4 μMol each of forward primer (5'-GTGCCTATTTGGTTATGACA-3') and reverse primer (5'-CTACTTACTAATCCTGCC CA-3'), 0.5 units Taq DNA polymerase, Recombinant (Invitrogen^{TM} life technologies, USA), and 1.5 mM of $MgCl_2$, in final volume of 25 μl. PCR conditions were an initial denaturation step of 5 min at 95° C, 40 cycles of 15 s at 94° C, 45 s at 55° C, 45 s at 72° C, and a final extension step of 10 min at 72° C. The amplified PCR products (286 pb) were digested with 2.5 U *Ase* I (New England Biolabs, Berverly, MA, U.S.A.) at 37° C. Digested products were analyzed on 3% agarose gel [28, 29]. The genotype was determined by digestion patterns of PCR products by *Ase* I (wild C allele: 286 pb, mutant T allele: 177+109 pb).

2.3 Statistical analysis

The chi-square or Fisher's exact tests were used for comparison of genotypes and allele frequencies among the ALL patients and the group of individuals without ALL. To estimate the relative risk of the lack of response to methotrexate in patients ALL associated with -401C/T and +452C/T genetic polymorphisms, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using logistic regression models. The Hardy-Weinberg test was used to determine the genetic equilibrium in group of individuals without ALL. The value of $p< 0.05$ was considered to be statistically significant. All statistical analyses were performed by using STATA software version 9.2.

3. RESULTS

3.1 General characteristics of children with ALL

We studied 70 children who suffered from ALL with a median age of 8 years. The predominant gender was male with 64.29% while there were 35.71% female patients. 92.86% of patients received a dose of MTX between 0.5-3 g/m2 weekly. 57.14% of the children with ALL had more than 1 year of treatment with MTX, while 68.57% had a relapse of ALL at some time of their treatment (Table 1).

3.2 General characteristics of children without ALL

We also included 140 children without ALL, in this group the range of age was from 1-18 years with a median age of 10 years, normal leukocyte count (4.0-10X10^{3}leucocytes/mm^3) and median of 7800 leukocytes/mm^3. In this group, the children were 53.57% male and 46.43% female (Table 1).

Table 1. General characteristics of the population and clinical data of children with acute lymphoblastic leukemia

Variable	ALL patients n= 70	Individuals without ALL n= 140
Age (years)	7.65 ± 4.67	9.99± 5.49
Number of leukocytes/mm^3	13000 (5400-39000)[a]	8000 (7000-9000)[a]
Sex		
Male	45 (64.29)	75 (53.57)
Female	25 (35.71)	65 (46.43)
Status of participants		
Live	30 (42.86)	140 (100)
Dead	40 (57.14)	0
Weekly MTX dose		
< 0.5 g/m^2	3 (4.29)	0
0.5- 3.0 g/m^2	65 (92.86)	0
> 3.0 g/m^2	2 (2.86)	0
Time on MTX		
< one years	30 (42.86)	0
> one years	40 (57.14)	0
Relapse during treatment with MTX		
No	22 (31.43)	0
Yes	48 (68.57)	0

Data indicate n (%); mean ± standard deviation (SD), [a] median (Percentiles 25-75)

3.3 Genotype distribution and allele frequency of -401C/T polymorphism of GGH in children with and without ALL

The most frequent genotype of the -401C/T polymorphism in both groups was the homozygous C/C which was found in 62.86% of the children with ALL and 75% of children without ALL. 17.14% of children with ALL and 21.43% of individuals without ALL presented the heterozygote genotype C/T, while the homozygous genotype T/T was observed in 20% of individuals with ALL and on 3.47% of individuals without ALL (Table 2). The genotypic frequencies in children without ALL were found to be in genetic equilibrium according to the Hardy-Weinberg Law (p = 0.1391). The observed frequency of allele T was 28.43% in children with ALL and 14.29% of children without ALL (Table 2).

Table 2. Genotype distribution and allele frequency of -401 C/T and +452 C/T polymorphisms of GGH in children with and without ALL.

Population (n)	Genotype distribution			*p*-value	Allele frequency		*p*-value	Hardy–Weinberg equilibrium
	C/C	C/T	T/T		C	T		
-401 C/T polymorphism								
ALL children (70)	44 (62.86%)	12 (17.14%)	14 (20.00%)		100 (71.43%)	40 (28.57%)		
Children without ALL (140)	105 (75.0%)	30 (21.43%)	5 (3.47%)	<0.001[a]	240 (85.71%)	40 (14.29%)	0.001[a]	**0.1391**
+452C/T polymorphism								
ALL children (70)	66 (94.29%)	4 (5.71%)	0 (0%)		136 (97.14%)	4 (2.86%)		
Children without ALL (140)	135 (96.43%)	5 (3.57 %)	0 (0%)	0.470[b]	275 (98.21%)	5 (1.79%)	0.489[b]	**0.8297**

[a] *p* value was obtained by the chi-square test. [b] *p* value was obtained by Fisher's exact test.

3.4 Genotype distribution and allele frequency of +452 C/T polymorphism of GGH in children with and without ALL

The most frequent genotype of the +452 C/T polymorphism of GGH in both groups was the homozygous C/C, which was observed in a 94.29% in cases with ALL and in the 96.43% of children without ALL, the heterozygote genotype C/T was observed in a 5.71% of individuals with ALL and a the 3.57% of individuals without ALL, whereas the homozygous genotype T/T was not observed in either group studied. The genotypic frequencies in children without ALL were found in genetic equilibrium according to the Hardy-Weinberg Law (p = 0.8297). The T allele was observed in 2.86% of individuals with ALL and in 1.79% of individuals without ALL (Table 2).

3.5 Genotype distribution and allele frequency of SNPs in children with ALL that are MTX responders or MTX non-responders.

The classification of children with ALL into MTX responders and MTX non-responders was based on the presence or absence of relapse of ALL after administration of MTX. The distribution of SNP -401C/T found was that 22.92% of children with ALL MTX non-responders had the C/T genotype, compared with 4.55% for the MTX responders, whereas 27.08% of the MTX non-responders were carriers of the T/T genotype compared to 4.55% of the MTX responders. The T allele was observed in a 38.54% of MTX non-responders and in 6.82% of MTX responders. There are statistically significant (p=0.004) differences between the MTX responders and MTX non-responders (Table 3).

Regarding the distribution of the +452C/T polymorphism, the C/T genotype was observed in 8.0%, while the T/T genotype was not observed in children who were MTX non-responders, compared with MTX responders in which both genotypes were not observed. The T allele was found in 4.17% of MTX non-responders and absent in MTX responders, so when comparing the genotypic distribution to the allelic frequency, we found that there is no statistically significant difference between the MTX responders and MTX non-responders (p=0.301 and p=0.308, respectively) (Table 3).

Table 3. Genotype distribution and allele frequency of -401 C/T and +452 C/T polymorphisms of GGH children with ALL responders and non-responders to MTX chemotherapy

	Responders' n (%)	Non-responders n (%)	*p**
-401 C/T			
Genotype			
C/C	20 (90.90)	24 (50.00)	
C/T	1 (4.55)	11 (22.92)	
T/T	1 (4.55)	13 (27.08)	0.004[a]
Allele			
C	41 (93.18)	59 (61.46)	
T	3 (6.82)	37 (38.54)	< 0.001[a]
+452 C/T			
Genotype			
C/C	22 (100)	44 (92.00)	
C/T	0 (0.00)	4 (8.00)	
T/T	0 (0.00)	0 (0.00)	0.301
Allele			
C	44 (100)	92 (95.83)	
T	0 (0.00)	4 (4.17)	0.308

* *p* value was obtained by Fisher's exact test. [a] Significant p<0.05.

3.6 Response to MTX based on the SNP -401C/T and +452C/T genotypes

We found a statistically significant association between C/T and T/T of the SNP -401(p = 0.042 and p = 0.028, respectively) with lack of response to MTX, children with ALL carrying the -401T/T genotype showed a significant increase in the lack of response to MTX (odds ratio 10.83, 95% CI 1.30-90.14) compared with those carrying the -401C/C genotype (Table 4).

Table 4. Association of -401 C/T polymorphism in GGH gene with the lack of response the MTX

	n	%	OR	CI 95%	*p*-value*
Genotypes					
C/C	44	62.86	1.00		
C/T	12	17.14	9.17	1.09-77.24	0.042
T/T	14	20.00	10.83	1.30-90.14	0.028

Odds ratio (OR); 95% confidence interval (CI); * *p* obtained by logistic regression analysis, taking the reference to CC genotypes.

The +452C/T SNP were not associated with lack of response to MTX, because no individuals of the group of responders were carrying the genotype CT or TT (data not shown). Other variables including age, sex, leukocytes count at diagnosis, and MTX dose, were not associated with the lack response to MTX (Table 5).

Table 5. Association the lack of response the MTX and the different covariables.

	n	%	OR	IC 95%	*p-value*
Sex					
Female	25	35.71	1.00		
Male	45	64.29	1.38	0.49-3.92	0.540[a]
Age (years)					
1-6	34	48.57	0.54	0 .19-1.50	0.236
7-12	24	34.29	1.60	0.53-4.83	0.405
13-18	12	17.14	1.46	0.35- 6.03	0.600
Leukocytes the diagnostic					
1000-10000	29	41.43	1.36	0.48-3.85	0.561
10001-100000	34	48.57	0.54	0.19-1.50	0.236
100001-290000	7	10.00	3.00	0.34-26.56	0.323
Weekly MTX dose					
< 0.5 g/m^2	3	4.29	0.91	0.08-10.64	0.942
0.5-3.0 g/m^2	65	92.86	0.52	0.06-4.98	0.574
> 3.0 g/m^2	2	2.86	0	0	0

Odds ratio (OR); 95% confidence interval (CI), [a] taking the reference to female sex

4. DISCUSSION

There have been attempts to explain the mechanisms by which patients show different response to the same drug used to treat ALL. The difficulty of explaining the response to drugs is due to the influence of genetic factors such as single nucleotide polymorphisms (SNPs) in genes encoding proteins and enzymes involved in the transport and metabolism of drugs, which have been associated with differences in the response to chemotherapy in several cancers [30]. However, there are few studies addressing the association of SNPs with response to methotrexate in patients with acute lymphoblastic leukemia. There are no studies in Mexico regarding the -401C/T and +452C/T polymorphisms of GGH, therefore, it was important to determine their distribution in the Mexican population and its association with the lack of response to MTX, due to the variation in response to MTX treatment in patients with ALL.

The analysis of the genotypic frequencies for the -401C/T polymorphism showed that the homozygous C genotype was the most frequent in children with ALL (62.86%), followed by the homozygous T genotype (20%), and the least frequent was the heterozygous C/T with 17.14%. These results differ (p=0.022) from that reported by Derveriux *et al*. in 2004 from the US population where they found that the frequency for the homozygous C genotype was 50%, 35% for the heterozygous C/T genotype and 16% for the homozygous T genotype [27].

While comparing the genotypic frequencies reported by Kim et al. in 2008 from the Korean population, where they reported a frequency of 66.5% for the homozygous C, 29.23% for the heterozygous C/T genotype and 4.62% for the homozygous T genotype [31], we found a statistically significant (p=0.013) difference, Mexican individuals showed a greater frequency of the -401C/T polymorphism of the GGH homozygous T genotype. This is probably due to the fact that both the US and Korean populations have different genetic background than the Mexican population, according to the National Mexican Institute for Genomic Medicine (INMEGEN), 65% of the genetics background of the Mexican population is not shared with any other population [32].

Regarding the +452C/T polymorphism of the GGH gene, the genotypic frequencies we found were significantly different (p=0.017) from those reported in the Caucasian population by Cheng *et al* in 2004. They found a frequency of 80.6% for the homozygous C genotype, 18.7% for the heterozygous C/T and 0.6% for the homozygous T genotypes [21]. In contrast, when comparing the Mexican frequencies with those reported for the Afro-American

population by Cheng *et al* in 2004, it was observed that there were no statistically significant differences (p=0.544). The frequencies reported were 91.3% for the homozygous C, 8.8% for the heterozygous C/T and 0.0% for the homozygous TT genotypes [21], this could be due to the fact that the population from the State of Guerrero in Mexico has 22% African genetic background according to the report by the INMEGEN in 2007 [32]. The finding in this study reflects in some way the ancestry of the population studied, in agreement with historical records indicating Guerrero and Veracruz States as the main entry points of African population during the Colonial period [33-35].

The -401C/T polymorphism has been associated with the increase in expression of GGH [15, 19]. Derveriux *et al* found that in patients with rheumatoid arthritis the individuals with the -401T/T genotype showed an increase in GGH activity that correlated with lack of response to MTX [27]. In this study, we found that those patients with ALL that had the -401 C/T heterozygous C/T genotype, were 9.17 ($CI_{95\%}$1.30-90.14) times more likely to relapse during treatment, while for those who had the T/T genotype it was 10.83 ($CI_{95\%}$1.3-90.14), compared to those patients that had the C/C genotype. These results were consistent with those reported by Chave et al, Yin et al and Derveriux et al.

The lack of response to MTX in patients with the +452C/T polymorphism could be due to the fact that this polymorphism alters the catalytic activity of the enzyme, causing it to accumulate PG-MTX, as shown in *in vitro* assays in B- ligeage cells from ALL [12].

Even though there is a close relation between the +452C/T polymorphism and GGH activity and MTX response, we did not find an association between the +452C/T SNP and the lack of response to MTX in patients with ALL, coinciding with that reported by Van der Straaten, where no association between the +452C/T SNP and clinical response to MTX in patients with RA was found [36]. Other variables such as sex, age, leukocyte count and MTX dose were not associated with the lack of response to MTX ($p>0.05$), which indicates that these variables did not have an effect on the response to chemotherapy in children with ALL, agreeing with that reported by Dulucq et al in patients with ALL, Dervieux et al in patients with RA and Kim et al in women with cervical cancer, who they found that these variable have no effect on the response to chemotherapy [31, 37, 38].

Our study suggests that the -401C/T polymorphism of GGH affects the response to MTX in patients with ALL but that is not the case for the +452C/T polymorphism. However,

further studies are needed to identify the polymorphisms associated with the pharmacokinetics of MTX and to define other genetic, karyotypic, and epigenetic factors to individualize MTX therapy in patients with ALL based on pharmacogenomics.

ACKNOWLEDGEMENTS

We would like to thank the National Council of Science and Technology (CONACYT, Mexico) for a fellowship awarded to Jorge Organista-Nava (August 2007 to July 2009). We thank everyone at the Instituto Estatal de Cancerología ‘‘Dr. Arturo Beltran Ortega’’ who helped with this study. We also thank technicians of the Laboratorio de Biomedicina Molecular for their laboratory assistance. We thank PhD. Elba Reyes-Maldonado (Morphology Department, National School of Biological Sciences, IPN), PhD. Gloria Fernández-Tilapa, M.Sc. Ma. Elena Moreno-Godínez, M.Sc. Eugenia Flores Alfaro, M.Sc. Adolfo Román-Román (Unidad Académica Ciencias Químico Biológicas, Universidad Autónoma de Guerrero) for critically reviewing this manuscript. Finally, we would like to thank Dinorah Leyva-Illades (Texas A&M Health Science Center) for revising the English style of the manuscript. The authors report no conflict of interest.

REFERENCES

1. Manuel J, Aranguré M, Carlos M, Álvarez O, Fajardo A. Epidemiologia de las Leucemias Agudas en niños. Parte 1*. Rev Med IMSS.2005; 43: 323-333.
2. González-Conejero R, Corral J, Roldán V, Vicente V. Farmacogenética en hematología. haematologica/edición española.2006; 91: 67.
3. Mejia-Arangure JM, Bonilla M, Lorenzana R, et al. Incidence of leukemias in children from El Salvador and Mexico City between 1996 and 2000: population-based data. BMC Cancer.2005; 5: 33.
4. Salud/INEGI Sd. Fuente: base de datos de defunciones INEGI/Secretaria de Salud, Dirección General de Información en Salud, México. Información disponible en URL www.inegi.org.mx/ [Acceso Abril 2008]
5. Cheok MH, Evans WE. Acute lymphoblastic leukaemia: a model for the pharmacogenomics of cancer therapy. Nat Rev Cancer.2006; 6: 117-129.
6. Fotoohi K, Jansen G, Assaraf YG, et al. Disparate mechanisms of antifolate resistance provoked by methotrexate and its metabolite 7-hydroxymethotrexate in leukemia cells: implications for efficacy of methotrexate therapy. Blood.2004; 104: 4194-4201.
7. Fotoohi AK. Resistance of Human Leukaemia Cells to the Antimetabolites. Oncology-Pathology, Suecia.2007; 1: 1-84.
8. Assaraf YG. Molecular basis of antifolate resistance. Cancer Metastasis Rev. 2007; 26: 153-181.
9. Hider SL, Bruce IN, Thomson W. The pharmacogenetics of methotrexate. Rheumatology (Oxford).2007; 46: 1520-1524.
10. Kager L, Cheok M, Yang W, et al. Folate pathway gene expression differs in subtypes of acute lymphoblastic leukemia and influences methotrexate pharmacodynamics. J Clin Invest.2005; 115: 110-117.
11. Gokbuget N, Hoelzer D. Treatment of adult acute lymphoblastic leukemia. Hematology Am Soc Hematol Educ Program.2006: 133-141.
12. Cheng Q, Cheng C, Crews KR, et al. Epigenetic regulation of human gamma-glutamyl hydrolase activity in acute lymphoblastic leukemia cells. Am J Hum Genet.2006; 79: 264-274.
13. de Jonge R, Hooijberg JH, van Zelst BD, et al. Effect of polymorphisms in folate-related genes on in vitro methotrexate sensitivity in pediatric acute lymphoblastic leukemia. Vol. 106: Am Soc Hematology.2005: 717-720.
14. Schneider E, Ryan TJ. Gamma-glutamyl hydrolase and drug resistance. Clin Chim Acta.2006; 374: 25-32.
15. Yin D, Galivan J, Ao W, Yao R. Characterization of the human gamma-glutamyl hydrolase promoter and its gene expression in human tissues and cancer cell lines. Gene.2003; 312: 281-288.
16. Dervieux T, Furst D, Lein DO, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis & Rheumatism.2004; 50: 2766-2774.
17. Urano W, Taniguchi A, Yamanaka H, et al. Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics.2002; 12: 183-190.
18. Wessels JAM, de Vries-Bouwstra JK, Heijmans BT, et al. Efficacy and toxicity of methotrexate in early rheumatoid arthritis are associated with single-nucleotide polymorphisms in genes coding for folate pathway enzymes. Arthritis & Rheumatism.2006; 54: 1087-1095.
19. Chave KJ, Ryan TJ, Chmura SE, Galivan J. Identification of single nucleotide polymorphisms in the human gamma-glutamyl hydrolase gene and characterization of promoter polymorphisms. Gene.2003; 319: 167-175.
20. Ansari M, Krajinovic M. Pharmacogenomics in cancer treatment defining genetic bases for inter-individual differences in responses to chemotherapy. Curr Opin Pediatr.2007; 19: 15-22.

21. Cheng Q, Wu B, Kager L, et al. A substrate specific functional polymorphism of human gamma-glutamyl hydrolase alters catalytic activity and methotrexate polyglutamate accumulation in acute lymphoblastic leukaemia cells. Pharmacogenetics.2004; 14: 557-567.
22. Hayashi H, Fujimaki C, Inoue K, Suzuki T, Itoh K. Genetic polymorphism of C452T (T127I) in human gamma-glutamyl hydrolase in a Japanese population. Biol Pharm Bull.2007; 30: 839-841.
23. Rhee MS, Wang Y, Nair MG, Galivan J. Acquisition of resistance to antifolates caused by enhanced gamma-glutamyl hydrolase activity. Cancer Res.1993; 53: 2227-2230.
24. Seguro-popular. Secretaria de Salud/ Seguro Popular, México 2009. Información disponible en URL http://www.seguro-popular.salud.gob.mx/ [Acceso Mayo 2009].
25. Merante F, Raha S, Reed JK, Proteau G. The Simultaneous Isolation of RNA and DNA from Tissues and Cultured Cells. Methods Mol Biol.1996; 58: 3-9.
26. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry.1987; 162: 156-159.
27. Dervieux T, Kremer J, Lein DO, et al. Contribution of common polymorphisms in reduced folate carrier and [gamma]-glutamylhydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. Pharmacogenetics.2004; 14: 733-739.
28. Ausubel FM, Brent R, Kingston RE, Moore DD eds. Current Protocols in Molecular Biology. Preparation and analysis of DNA. Vol. 1. United States of America; 1994-2000.
29. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. Vol. 1-2 (ed Third): Cold Spring Harbor Laboratory Press; 2001.
30. Efferth T, Volm M. Pharmacogenetics for individualized cancer chemotherapy. Pharmacology and Therapeutics. 2005; 107: 155-176.
31. Kim K, Kang SB, Chung HH, Kim JW, Park NH, Song YS. XRCC1 Arginine194Tryptophan and GGH-401Cytosine/Thymine polymorphisms are associated with response to platinum-based neoadjuvant chemotherapy in cervical cancer. Gynecologic Oncology. 2008. 111: 509-515
32. INMEGEN. Instituno Nacional de Medicina Genomica. Mapa genetico de los mexicanos. México, 2007. Información disponible en URL http://www.inmegen.gob.mx/ [Acceso Abril 2009].
33. Gorodezky C, Alaez C, Vázquez-Garci´a MN, et al. The genetic structure of Mexican Mestizos of different locations: tracking back their origins through MHC genes, blood group systems, and microsatellites. Human Immunology.2001; 62: 979-991.
34. Wang S, Ray N, Rojas W, et al. Geographic Patterns of Genome Admixture in Latin American Mestizos. PLoS Genet.2008; 4: e1000037.
35. Silva-Zolezzi I, Hidalgo-Miranda A, Estrada-Gil J, et al. Analysis of genomic diversity in Mexican Mestizo populations to develop genomic medicine in Mexico. Proceedings of the National Academy of Sciences.2009; 106: 8611-8616.
36. van der Straaten R, Wessels JAM, de Vries-Bouwstra JK, et al. Exploratory analysis of four polymorphisms in human GGH and FPGS genes and their effect in methotrexate-treated rheumatoid arthritis patients. Pharmacogenomics.2007; 8: 141-150.
37. Dulucq S, St-Onge G, Gagne V, et al. DNA variants in the dihydrofolate reductase gene and outcome in childhood ALL. Blood.2008; 111: 3692-3700.
38. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis & Rheumatism.2006; 54: 3095-3103.